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(54) Method for detecting a target nucleic acid sequence.

(57) Target nucleic acid is detected by reorganizing an excess of two complementary pairs of single stranded probes, which hybridize to contiguous target sequences. Nucleic acid in the sample is annealed to the probes, and contiguous sequences are ligated to form complementary detectable fused probes complementary to the original target, and the fused probes serve as a template for further fusions. The reorganized species being detected is increased at a geometric rate by cycles of annealing probes to the target, ligating the annealed probes in a template-dependent manner, and separating the fused probes from the template to form new templates.

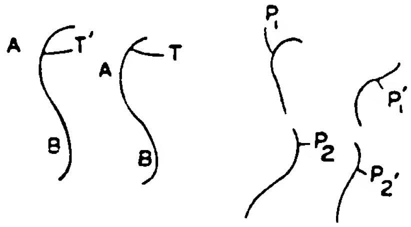

FIG. 1A

EP 0 320 308 A2

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Description

DETECTING TARGET NUCLEIC ACID

This invention relates to the detection of nucleic acid.

Nucleic acid hybridization has been proposed to detect the presence of a particular nucleic acid in a sample For example, Falkow U.S. Patent No 4,358,535 discloses a hybridization assay in which single-stranded DNA is attached to a filter, labeled, single-stranded sample DNA is contacted with the filter, and hybridization between sample DNA and the labeled, hybridized probe is detected on the filter

Whiteley et al EP 185494 discloses detecting a target nucleic acid sequence that has a diagnostic portion, by treating the sample with a probe complementary (under low stringency conditions) to the diagnostic portion and then treating the sample with a probe complementary (under high stringency conditions) to a contiguous sequence The diagnostic and contiguous probes are covalently attached, and the attached probes are detected after unattached probes are removed.

Mullis U S. Patent Nos. 4,683.202 and 4,683,195 disclose a process for amplifying a nucleic acid sequence by treating complementary nucleic acid strands with primers and extending the primers using DNA polymerase to form a template for synthesizing the desired nucleic acid. The '195 patent features detecting DNA that has been amplified by that process.

We have discovered a method for detecting the presence and abundance of a target nucleic acid sequence in a sample. The method involves rapid cyclic template-dependent reorganization of an excess of probe sequences at a geometric rate, thereby rapidly increasing the availability of the sequence being detected and ultimately increasing the sensitivity of the assay. The use of this method is particularly advantageous when the target sequence is present in low levels, or when it is an extremely minor component in a sample containing other nucleic acid sequences. The process can be readily adapted to automation making it particularly attractive for use in diagnostic kits.

The invention generally features, in one aspect thereof, detecting a target nucleic acid sequence in a sample using a stoichiometric excess of at least four single stranded nucleic acid probes. For convenience, the first and second probes will be called primary probes, and the third and fourth probes will be called secondary probes. The probes have the following characteristics. The first probe is capable of hybridizing to a first segment of a strand of the target nucleic acid sequence, and the second probe is capable of hybridizing to a second segment of the same strand of the target nucleic acid sequence. The first and second probes are selected to enable joining of the 3' end of the first probe to the 5' end of the second probe, when the two probes are hybridized to the target sequence, --i.e., the 5' end of the first segment of the target sequence strand is positioned relative to the 3' end of the second segment of that strand to enable joining of the probes. The first probe is also hybridizable to the third probe, and the second probe is hybridizable to the fourth probe.

The assay works as follows in a preferred procedure:

Sample DNA is provided as single-stranded DNA, including two complementary target strands (a primary target strand and a secondary target strand) if the target is double stranded The four probes are introduced to the sample DNA as four single strands so that the two primary probes hybridize to the primary target strand, and (if the target is double-stranded) the two secondary probes hybridize to the secondary target strand. Next, the primary probes are ligated, forming a primary synthetically fused probe sequence, and (for double-stranded targets) secondary probes are fused forming a secondary synthetically fused probe sequence. The DNA is denatured, in effect doubling the target population in the sample As the cycle of hybridization, ligation and denaturation is repeated, the population of reorganized detectable fused probes increases at a geometric rate. Where the target is single-stranded, the secondary probes lack a target strand until the second cycle, at which point the primary synthetically fused probe sequence forms a template for the two secondary probes, and the assay proceeds as described above. The technique enables reorganization of the probe sequence, to form the fused probe sequence(s) being detected, at a geometric rate in accordance with the principles described below. Rapid reorganization provides excellent sensitivity, using a simple protocol. Preferably, the cycle is repeated 20-50 times.

It is also preferred that the 5' end of the first section of the primary target strand abuts (is contiguous with), and is joined by a phosphate bond to, the 3' end of the second section of the primary strand target, without any intervening sequences, to provide efficient ligation, particularly enzymatic ligation. DNA is the preferred nucleic acid, both for the probes and for the target. The preferred method of separating complementary sequences is by heat denaturation, i.e., melting. Preferably the probes are 10-200 bases long. Additional (fifth, sixth, etc.) probes can be used which hybridize adjacent to the other probes and can be joined to those probes in the same way. However, four probes are sufficient and preferred.

The above described method can be used with sensitive detection systems, particularly systems involving a combination of labeling entities on two different probes. For example, the labeling entity on one probe can be a specific binding partner for an insoluble phase (e.g biotin for an avidin-functionalized insoluble phase), and the labeling entity on the other probe can be a chromophore or fluorophore. After the insoluble phase has been exposed to the sample and washed, the presence of chromophore or fluorophore on that phase indicates the presence of synthetically fused probe, and thereby indicates the presence of target in the sample.

The invention also features, in an alternative aspect thereof, a kit for performing the assay, including the probes, the ligase, and means to separately contain the probes and the ligase. Apparatus for performing the method includes means to hold a mixture comprising the target sequence, probes and ligase, and means to cycle the temperature of the mixture from a denaturing temperature to a temperature allowing hybridization of the probes to the target. Preferably, the temperature is cycled automatically.

Other features and advantages can be apparent from the following description of the preferred embodiments. In the drawings:

Fig. 1 is a diagrammatic representation of steps in a hybridization assay; and

Fig. 2 is a graph depicting formation of reorganized probes being detected as a function of cycle number.

The invention is illustrated by Fig. 1, which depicts steps in a hybridization assay for detecting a nucleotide sequence present in low concentrations.

Those skilled in the field will recognize that there are numerous ways to perform various steps in the method. Generally, the steps can be performed using well-known techniques such as those described in Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory (1982). For example, double-stranded DNA can be rendered single-stranded by heat denaturation ("melting") at 80°C - 105°C for 1-5 minutes. Alternatively, enzymatic strand separation can be used. Probes or sub-segments can be synthesized using standard techniques for synthesizing oligonucleotides, or by digesting naturally occurring DNA and isolating fragments. Hybridization conditions will depend on the length and degree of homology of the fragments involved. Generally, the technique and conditions described by Wetmar et al. J. Mol. Biol. 31:349-370 (1968) can be used. Appropriate conditions and techniques for using nucleotide ligases are well known and are supplied by the manufacturer.

Certain features of this system, while not essential, are preferred. In particular, only the 5' ends that participate in template-dependent joining should be phosphorylated by standard techniques, if they are not already phosphorylated, so as to suppress joining involving other 5' ends. The lengths and sequences of probes are selected so that, should an incorrect joining of two probes occur (i.e. should two probes join in a manner not represented by a linear sequence on the intended target) those incorrectly joined probes will not serve as a template for the joining of their complementary probes, because the ends of the complementary probes will not be adjacent to each other on a proper manner for enzymatic ligation. Preferably, the probes are between 10 and 200 bases long.

Preferred ligases are those that do not tend to catalyze template independent joining of the probes under at least one set of reaction conditions which is otherwise suitable for the procedure. For example, satisfactory results are achieved with E. coli DNA ligase (available from U.S. Biochemical) or T. thermophilus DNA ligase in the absence of high concentrations of volume excluding solutes, or with T4 DNA ligase on the presence of about 5.0 mM ATP. See, Zimmerman et al., Proc. Nat'l. Acad. Sci. 80, 5852 (1983); Takahashi, M., Uchida, T. J. Biochem. 100, 123 (1986); and Ferreti et al., Nuc. Acids Res. 9, 3695 (1981).

It is also preferred that the ligase enzyme not be denatured by the step intended to dissociate duplex DNA into its constituent strands. Where denaturation is accomplished by increasing the temperature, a thermo-stable ligase is preferable. The benefits of such an enzyme include decreased reagent cost, decreased operating complexity, reduction of amount of undesirable components added (the enzymes are often stored in buffers containing glycerol), and potentially greater shelf life for the reagents. The preferred thermostable ligase is ligase from Thermus thermophilus (e.g. ATCC 27634) purified by the general technique of Takahashi et al., J. Biol. Chem. 259, 10041 (1983).

Fig. 1 shows a hybridization assay detecting a double-stranded target DNA sequence, represented by T-T'. The target sequence is present in a sample containing many unrelated DNA sequences.

The assay features a kit containing two complementary pairs of probes, represented by P_1-P_1' and P_2-P_2', in a standard solution. These probes are selected to be complementary to various portions of the target sequence. Specifically, P_1 is complementary to segment A of strand T; P_2 is complementary to segment B of strand T; P_1' is complementary to segment A of strand T'; and P_2' is complementary to segment B of strand T'. The probes are selected to be long enough to provide selective hybridization, and to generate a fusion sequence that is readily distinguished from other sample components. We have found that probes of 10-200 bases are satisfactory. Most preferably, the probes are between 12 and 50 bases. The probes are provided in large excess to drive the reactions described below. For example, the probe concentration preferably is between about 10^{12} and 10^{14} molecules per 50 µL reaction volume.

One cycle of the method is illustrated by Figs. 1A-1D. First (Fig. 1A), the sample DNA is denatured. Then hybridization is permitted (Fig. 1B). If T is present in the sample, there is a relatively high likelihood that T will encounter P_1 and P_2, and form the species indicated in Fig. 1B. Similarly, T' will encounter P_1' and P_2'.

The next step in the cycle is addition of a ligase that will ligate the adjacent probe ends (Fig. 1C), but generally will not ligate blunt ends of DNA in the sample. After ligation, the sample is subjected to denaturing conditions (Fig. 1D), yielding the fused probes P_1-P_2 and P_1'-P_2'. From that point, the sanple is ready for a new cycle of hybridization-ligation-denaturation.

As will be seen from this example of one cycle, the sample increases from one double-stranded template T_1-T_1' at the beginning of the cycle to two double stranded templates. Assuming ideal efficiency in the next cycle, each of these two synthetic double-stranded templates, as well as the original target, will yield two double-stranded templates. Table 1 shows this progression for n cycles, where X is the number of T-T_1 pairs before cycle 1.

Table 1

No of Cycles	No of P_1-P_2	No of P_1'-P_2'
1	1 • X	1 • X
2	3 • X	3 • X
3	7 • X	7 • X
4	15 • X	15 • X
n	$(2^n-1)X$	$(2^n-1)X$

Since the species P_1-P_2 (and, if desired P_1'-P_2') is detectable, repeated cycles improve detection sensitivity, up to a point. For each cycle, there is a very small but finite chance of forming P_1-P_2 or P_1'-P_2' by blunt end ligation in the absence of T or T'. Once this event occurs, the ligated species is indistinguishable from the presence at the outset of T or T'. Limiting the number of cycles reduces the opportunity for such a false positive reading. Also, at some point the unfused probes are depleted to a level that cannot drive the desired reaction, and there is less chance that fused probes will hybridize with unfused probes (as opposed to the unproductive hybridization of two fused probes).

Fig 2 shows curves depicting the number of detectable fused probes present in the mixture as a function of the number of cycles. Depending on X (the number of target probes originally present), the number of reorganized fused probes will increase geometrically according to the above equation, up to some level at which the rate of increase slows dramatically. By plotting this relationship against standards, and determining how many cycles are required to reach a given level, it is possible to determine the quantity of target present initially.

C Example 1

Four deoxyribonucleotide oligomers were prepared by standard methods. The oligomers had the following sequences:

P_1 = 5' GCGGATCCTCTAGAGTCGACCTGCA 3'
P_2 = 5' AATTCGAGCTCGGTACCC 3'
P_1' = 5' GGTCGACTCTAGAGGATCCCC 3'
P_2' = 5' GGGTACCGAGCTCG 3'

P_1 and P_2 are abutting sequences on one strand of the polylinker region of the plasmid pUC18, and plasmids P_1' and P_2' are abutting sequences on the complementary strand.

Primers P_1' and P_2 were treated with polynucleotide kinase and ATP to render their 5' ends phosphorylated. Primer P_1 was radioactively labeled at its 3' end by treatment with terminal transferase and α-^{32}P-dCTP.

D Example 2

Samples were prepared which contained 30mM TrisCl pH8.0, 100 mM NaCl, 1.2mM EDTA, 4.0mM $MgCl_2$, 1.0mM dithiothreitol, 50μ/ml Bovine Serum Albumin, 20μg/ml of Hela DNA plus 20μg/ml nonspecific oligonucleotide DNA (e.g., the following 20 mer: 5'-ATCGATACATCAGGAATATT-3'), 1μg/ml of each of the probes of Example 1 and various amounts of pUC18 plasmid DNA linearized at the EcoRI cleavage site. 50μl aliquots of these samples were subjected to the following steps:

(a) heat to 100°C for 1 minute to denature the DNA

(b) incubate at 37°C for 1 minute to allow DNA renaturation

(c) add 50 units *E. coli* DNA ligase (using units defined by the manufacturer, United States Biochemical Corporation)

(d) incubate at 37°C for 1 minute to allow joining of appropriately juxtaposed probes

Steps (a) through (d) were repeated between 20 and 50 times. Aliquots were removed, treated to destroy residual ligase activity, and saved. The saved aliquots were analyzed by polyacrylamide gel electrophoresis and autoradiography. The time of appearance (in number of cycles) of detectable quantities of joined material strongly correlates with the number of target molecules initially present in the reaction.

Other embodiments are feasible.

For example, RNA can be used as well as DNA. In the examples, Hela DNA and a nonspecific oligonucleotide were included to protect the probe from degradation by nucleases that might be present in the sample. However, these are not essential.

Claims

1. A method of detecting target nucleic acid in a sample comprising the steps of:

(a) providing nucleic acid of the sample as single-stranded nucleic acid;

(b) providing in the sample at least four nucleic acid probes, wherein: i) the first and second of said probes are primary probes, and the third and fourth of said probes are secondary nucleic acid probes; ii) the first probe is a single strand capable of hybridizing to a first segment of a primary strand of the target nucleic acid; iii) the second probe is a single strand capable of hybridizing to a second segment of said primary strand of the target nucleic acid sequence, iv) the 5' end of the first segment of said primary strand of the target is positioned relative to the 3' end of the second segment of said primary strand of the target ot enable joining of the 3' end of the first probe to the 5' end of the second probe, when said probes are hybridized to said primary strand of said target nucleic acid; v) the third probe is capable of hybridizing to the first probe; and vi) the fourth probe is capable of hybridizing to the second probe; and

(c) repeatedly performing the following cycle:

i) hybridizing said probes with nucleic acid in said sample.

ii) ligating hybridized probes to form reorganized fused probe sequences, and

iii) denaturing DNA in said sample, and

d) detecting the reorganized fused probe sequences;

whereby with successive cycles the quantity of reorganized fused primary and fused secondary probes is increased.

2. A method according to Claim 1, wherein the 5' end of the first segment of said primary strand of the target sequence abuts, and is joined by a covalent bond to, the 3' end of the second segment of said primary strand of the target sequence, without intervening bases.

3. A method according to Claims 1 or 2 wherein the probes are joined by an enzyme.

4. A method according to Claim 3, wherein the probes are joined by a ligase, preferably a bacterial ligase.

5. A method according to Claim 4, wherein the ligase is *Escgerichia coli* DNA ligase or *Thermus thermophilus* DNA ligase.

6. A method according to any preceding claim, wherein the nucleic acid probes are DNA.

7. A method according to any preceding claim wherein the target nucleic acid sequence is DNA.

8. A method according to any preceding claim, wherein the fused nucleic acid is separated from the target sequence by heat denaturation.

9. A method according to any preceding claim, wherein said cycle is repeated at least twice, preferably between 20 and 50 times.

10. A method according to any preceding claim, wherein the 5' end of the second probe but not of the first probe is phosphorylated.

11. A method according to any preceding claim, wherein the target sequence is double-stranded before step (a).

12. A method according to any preceding claim, wherein at least one of said probes is labelled with a labelling entity.

13. A method according to Claim 12, wherein both of said primary probes are labelled with a labelling entity.

14. A method according to Claims 12 or 13, wherein both of said secondary probes are labelled with a labelling entity.

15. A method according to any of Claims 12, 13 or 14, wherein the or at least one said labelling entity comprises a chromophore or flurophore.

16. A method according to any of Claims 12, 13 or 14, wherein the or at least one said labelling entity comprises a specific binding partner for an insoluble phase.

17. A kit for performing an assay in accordance with any preceding claim comprising said probes, a nucleic acid ligase, and means adapted to contain said probes separately from said nucleic acid ligase.

18. Apparatus for performing a method in accordance with any of Claims 1 to 16, comprising means adapted operatively to hold a mixture comprising said target sequence, said probes and a ligase, and means adapted operatively to cycle the temperature of said mixture between a first temperature that denatures nucleic acid in said sample and a second temperature allowing hybridization of the probes to the target.

19. Apparatus according to Claim 18, wherein said means adapted to cycle temperature comprises means adapted operatively to vary said temperature automatically.

20. For use in a method of detecting target nucleic acid in a sample, a set of at least four nucleic acid probes as defined in any of Claims 1 to 16.

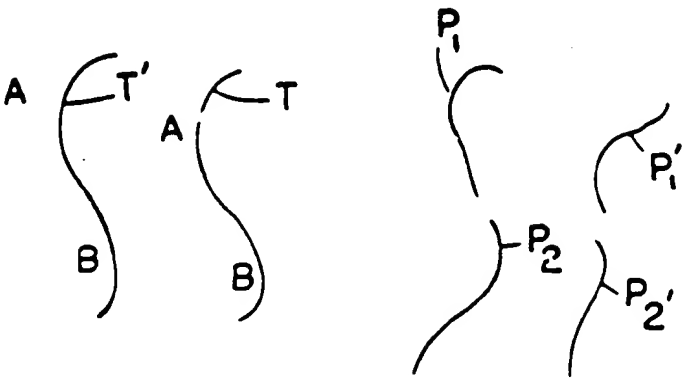

A
T'
A
T
B
B
P1
P1'
P2
P2'

FIG. 1A

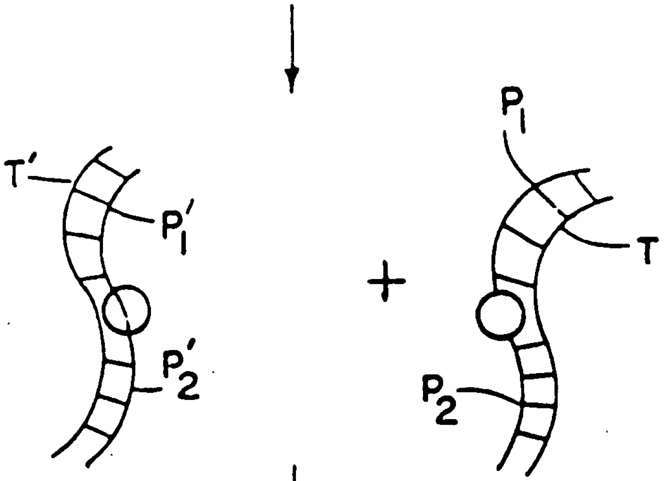

T'
P1'
P2'
+
P1
T
P2

FIG. 1B

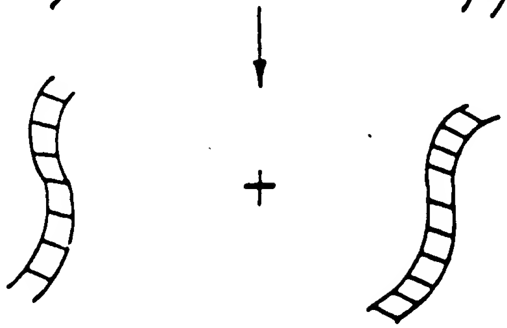


FIG. 1C

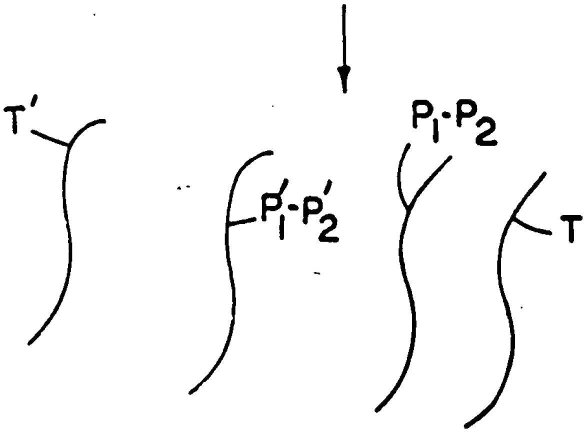
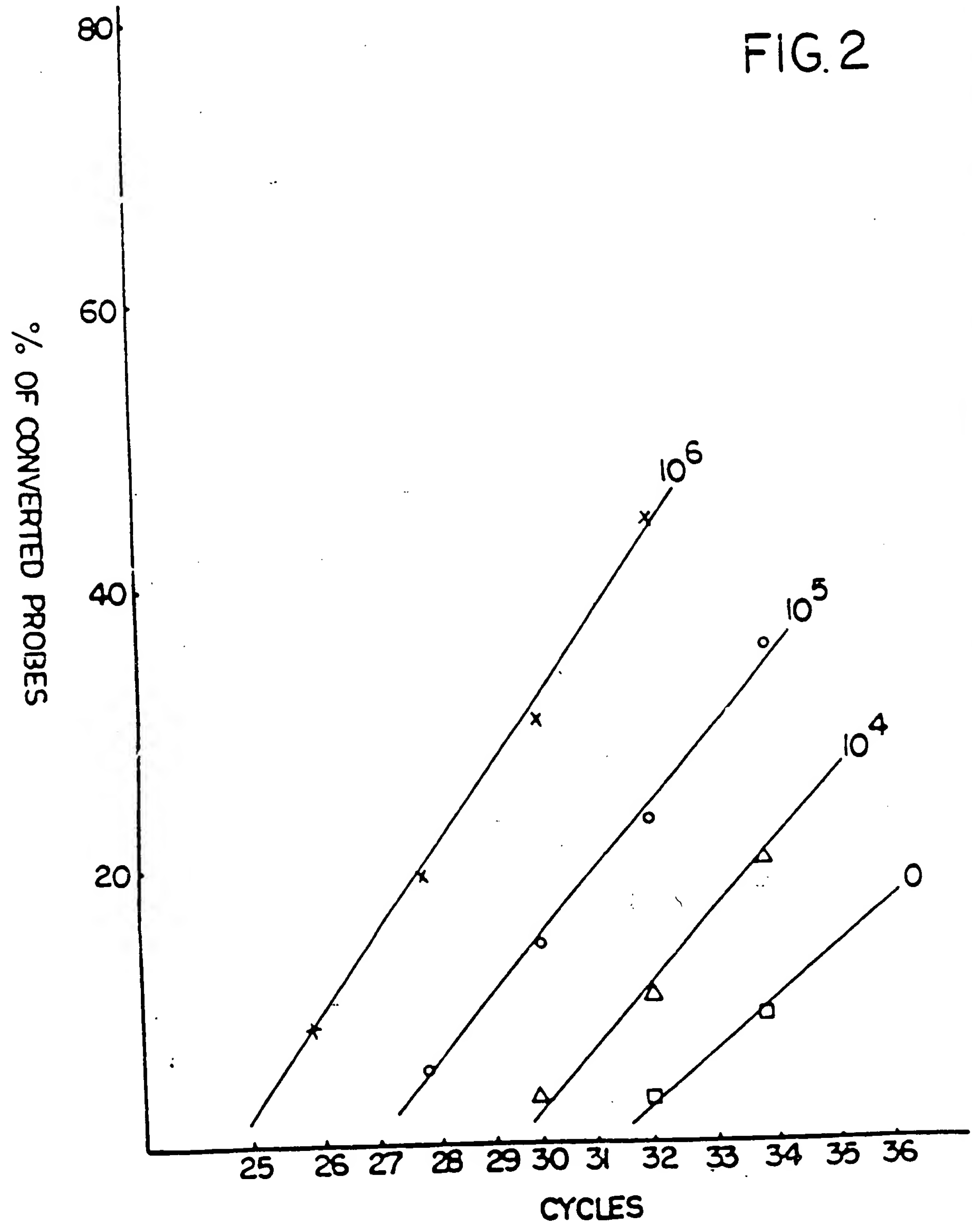

T'
P1'-P2'
P1-P2
T

FIG. 1D

FIG. 2


% OF CONVERTED PROBES
80
60
40
20
25
26
27
28
29
30
31
32
33
34
35
36
CYCLES
10^6
10^5
10^4
0